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Joint Research Entitled

Threshold Concentrations of Molecular Hydrogen Allowing for Growth of Methanogenic Bacteria

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In 1976, the Viking Lander missions analyzed the Martian soil for evidence of life. While the results led to some controversy, most investigators concluded that there was no biological activity at the lander sites. But the Viking Landers only examined the surface of Mars where conditions are too dry, too cold, and too oxidizing for life to exist as we know it. It may be possible that liquid water can exist and persist below the surface. There is evidence that liquid water has been present in subsurface aquifers on Mars throughout its history. Lifeforms existing below the surface could not obtain their energy from photosynthesis, but rather they would have to utilize chemical energy. Because the Viking Landers found no measurable quantities of organic matter, lifeforms might be limited to oxidation of inorganic matter for energy. Organisms that fall into this category are referred to as chemoautotrophs. Methanogens are chemoautotrophs that consume molecular hydrogen and carbon dioxide and produce methane as a waste product. A potential habitat for existence of methanogens on Mars might be based on a geothermal source of hydrogen, possibly due to volcanic or hydrothermal activity, or the reaction of basalt and anaerobic water,

carbon dioxide, which is already abundant in the Martian atmosphere, and subsurface liquid water.

Since carbon dioxide is abundant, and molecular hydrogen might be a limiting nutrient, experiments were designed to determine minimum threshold concentrations of molecular hydrogen under various conditions of temperature, pressure, pH, and nutrient concentrations that will allow for growth of the methanogen tested.

All organisms were grown in a standard medium (MS Medium) for methanogens or the same medium without organic material. Growth medium was prepared under 95% carbon dioxide and 5% hydrogen in a Coy environmental chamber. A carbon dioxide rather than the standard nitrogen atmosphere was preferable. Medium made in the described atmosphere was saturated with carbon dioxide resulting in a pH of 6.7 which is ideal for most methanogens. A nitrogen atmosphere would increase the medium pH to greater than 8, resulting in precipitation of medium components and unfavorable conditions for growth. The 5% hydrogen was necessary for the oxygen-removing palladium catalysts to work. (Methanogens are strict anaerobes and are poisoned by molecular oxygen.) The anaerobically-prepared medium was added to growth vessels in the

anaerobic chamber. Each growth vessel was sealed with a butyl rubber stopper and a crimped aluminum cap. Outside of the anaerobic chamber, the gas phase in each vessel was replaced with100% hydrogen by the use of a gassing manifold. The combination of the hydrogen headspace gas along with a carbon dioxide-saturated liquid medium resulted in a combination of substrates ideal for methanogenic growth. In experiments where cultures needed to be repressurized, a 75% hydrogen: 25% carbon dioxide mixture served the same purpose. At least one hour prior to inoculation, a sterile sodium sulfide solution was added to each culture tube to eliminate any residual molecular oxygen. A sterile 3cc plastic syringe was used to inoculate each vessel. Growth was monitored spectrophotometrically using a Spectronic 20 at 660nm. When each culture reached an optical density of 0.1 to 0.2, the headspace was flushed and replaced with pure carbon dioxide (300 kPa total pressure). Hydrogen was then be added to provide 10,000 to 20,000 ppm in the headspace. Headspace gas samples (1ml) were removed at time intervals and analyzed by a Hewlett Packard model 5890 gas chromatograph with a thermal conductivity detector at an oven temperature of 40C using argon as the carrier gas.

Previous studies have shown that methanogens can consume H_2 down to levels of 22 ppm when provided with a source of organic material.

Thus far, research in our laboratory has resulted in the submission of a manuscript entitled "Hydrogen Consumption by Methanogens on the Early Earth" (see attached manuscript). Besides myself, the authors include Keith Brink (graduate student), Stanley Miller who was responsible for the thermodynamic calculations and ensuing discussion, and Chris McKay, our research collaborator from NASA Ames Research Center, Moffett Field, CA. Even though the original impetus for the research dealt with the possibility of life on Mars, the results have significant implications for early life on Earth.

To determine the lower limit at which chemoautotrophic methanogens can consume H_2 we have conducted tests on several methanogens. In the manuscript we report results for *Methanobacterium wolfei*, *Methanobacterium formicicum*, and *Methanosarcina barkeri*. For all three methanogens considered there is a clear and reproducible limit on the levels of H_2 where uptake occurs. For growth on inorganic medium, these levels vary among the organisms tested with the lowest value, 10+/-10 ppm, corresponding

to *M. formicicum*. *M. wolfei* demonstrated a minimum value of 49+/-21 ppm, while the highest value of the H_2 uptake level was 157+/-21 ppm for *M. barkeri*. Growth on organic medium resulted in values of 121+/-24 ppm for *M. barkeri*, 61+/-19 ppm for *M. wolfei*, and 12+/-8 ppm for *M. formicicum*.

Our results for heterotrophic growth can be compared to those of Lovley and colleagues. For example, they found that *M. formicicum* in pure culture would take up H_2 to a partial pressure of 6.5 Pa (equivalent to 22 ppm in our experiments). Studies on whole sediments also show uptake of H_2 down to levels of 7-10 nM in solution (equivalent to 3-5 ppm in our experiments).

The calculated free energies for the methanogens are

M. barkeri at 37C on inorganic medium, ΔG_{react}=-14.64 kcal

M. barkeri at 37C on organic medium, ΔG_{react} = -14.10 kcal

M. formicicum at 37C on inorganic medium, ΔG_{react} = -7.51 kcal

M. wolfei at 55C on inorganic medium, ΔG_{react} = -8.96 kcal

The last two ΔG values seem a little low if several kcal of irreversibility are needed, but the $\Delta G°$ of hydrolysis of ATP is rather dependent on Mg^{+2}, Ca^{+2}, temperature and the ATP/ADP ratio. It is also possible that the ATP is produced by a chemiosmotic pathway

from the free energy obtained from the reduction of two CO_2 molecules, although this does not seem to be required in this case. It is noted that formate has an equivalent pH_2 of nearly 1 atm so that traces of formate in the medium could provide considerable free energy in addition to that provided by the H_2.

Following completion of this manuscript, hydrogen thresholds for two other methanogens have been determined. *Methanococcus maripaludis* demonstrated a threshold of 27+/-11 ppm in organic medium and 30+/-12 ppm in inorganic medium. *Methanococcus voltae* demonstrated a threshold of 97+/-28 ppm in organic medium. We have been unable to grow *M. voltae* in inorganic medium so far. The free energy changes are -12.21, -12.45, and -13.06 kcal, respectively.

Additionally, the hydrogen threshold of a psychrophilic methanogen isolated from Ace Lake in Antarctica is being determined. This organism, which has been maintained at 7C, is currently demonstrating a hydrogen threshold of no greater than 140 ppm. Of course, this organism is very interesting to us because of the correlation between its cold-loving characteristic and the

extremely low temperatures on Mars. A complete characterization of this organism is in progress.

Once the hydrogen thresholds had been determined under "standard" growth conditions for the methanogens described above, we set our next goal as determining the hydrogen thresholds under "non-standard" conditions of temperature, pH, medium composition, and pressure. Since conditions on Mars and early Earth are typically considered extreme, we wanted to determine hydrogen thresholds under extreme conditions. However, before examining hydrogen thresholds, we had to determine the effects of the extreme conditions on the growth of the methanogens.

In order to determine the effects of pH on both growth and hydrogen threshold, medium was altered by the addition of hydrochloric acid or sodium hydroxide. Molecular nitrogen can be used to increase the pH, but achieving a predetermined stable pH is difficult. The pH of the unaltered medium is 6.7. The pH was adjusted in 0.5 pH intervals. Initial results indicate that *M. wolfei* prefers to grow in slightly acidic rather than basic medium. The organism generated 19% methane at pH 6.7, 15% at pH 6.2, and 7.8% at pH 5.7.

At pH levels of 7.2 and 7.7, *M. wolfei* generated only 0.69% and 0.06% methane, respectively.

Pressure experiments were also conducted. A gassing manifold with an oxygen scrubber was used to deliver pressures of 20 (-80 relative to ambient pressure), 100, 200, and 300 kPa. All of the methanogens tested so far prefer to grow at high pressure. (These results are not surprising due the the low solubility of H_2.) Growth is greatest at 300 kPA, followed by 200 kPa, and then100 kPa. These organisms have not demonstrated growth in preliminary experiments at 20 kPa so far.

Temperature experiments have demonstrated the obvious, maximal methane production at the optimal temperature. In addition, and maybe not so obvious, growth at higher or lower than optimal temperatures has resulted in variable results, depending on the organism. As an example, *M. maripaludis* which grows optimally at 37C produced a fair amount of methane at 27C, but very little at 55C. *M. formicicum*, again with optimal growth at 37C, produced very little methane at either 27C or 55C.

Experiments to determine the requirement for or stimulatory effects of various mineral supplements were also conducted.

Preliminary results with *M. wolfei* indicate that none of the trace minerals included in the standard MS mineral medium are required. This is in disagreement with at least one literature article which suggests that tungsten is required by *M. wolfei*. Also, growth has occurred in the absence of added magnesium or calcium. As a matter of fact, growth appears to be better without these additions. However, it must be noted that there are trace mineral contaminants in all of the other medium components, especially iron. We are currently working on this problem.

Once we get a handle on the optimal growth conditions (temperature, pH, pressure, and nutrients) and the effects of altering those conditions on the growth of selected methanogens, we will begin to investigate the hydrogen thresholds under those altered conditions.

In addition to the manuscript referred to earlier, a grant proposal has also been drafted and will be submitted to the Life in Extreme Environments Program at NSF. (See attached copy of the proposal.)

In addition to the manuscript and grant proposal, two poster presentations were delivered at scientific meetings. The first was presented at a Gordon Conference (Origins of Life) in January of 1996. The abstract is below.

HYDROGEN CONSUMPTION BY METHANOGENS ON THE EARLY EARTH.
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It is possible that the first autotroph used chemical energy rather than light. This could have been the main source of primary production after any initial inventory of abiotic organic material had been depleted. The electron acceptor most readily available for use by the first chemoautotroph would have been CO_2. The electron donor available may have been H_2 that would have been outgassing from volcanoes at a rate estimated to be as large as 10^{12} moles/year. Methanogenic microorganisms, classified within the domain Archaea, are known to utilize these compounds. They may have had a profound effect on the chemistry of the early atmosphere by producing CH_4 and limiting the buildup of H_2. We report that certain methanogens will consume H_2 down to levels as low as 10 ppm with CO_2 (at three atmospheres pressure) as the sole carbon source. H_2-consuming autotrophs are likely to have been a dominant sink for H_2 on the early Earth. It has also been suggested that a microbial ecosystem based on H_2 and CO_2 could be the basis for subsurface life on Mars where CO_2 is abundant, but surface conditions are inhospitable to life. Subsurface heat sources could provide liquid water and H_2. Alternatively, H_2 could be produced by water reacting with basalt, which is abundant on Mars, as is seen in certain terrestrial ecosystems.

The second poster was presented at the American Society for Microbiology annual meeting in May of 1996. Its abstract is below.

Molecular Hydrogen Consumption and Growth Characteristics of Three Methanogens Grown Under Hypothetical Mars-Like Conditions.
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Even though the Viking Lander missions in 1976 found no evidence of life on the surface of Mars, investigators continue to believe that life may exist in subsurface habitats. A subsurface source of molecular hydrogen, along with carbon dioxide, which is already abundant in the Martian atmosphere, might support the growth of methanogenic microorganisms. This research examines the molecular hydrogen consumption and growth characteristics of *Methanobacterium wolfei* , *Methanobacterium formicicum, and Methanosarcina barkeri* under conditions that might mimic subsurface habitats on Mars. MS Medium (organic) and Mineral Medium (inorganic) were prepared in an anaerobic chamber and distributed into special anaerobic tubes. Each culture tube was inoculated with a single species of methanogen and pressurized with a mixture of 75% hydrogen and 25% carbon dioxide. Molecular hydrogen consumption was monitored by gas chromatography. For all three methanogens considered, there is a clear and reproducible limit on the levels of hydrogen where uptake occurs. These levels vary among the organisms and the media tested with the lowest value, 10 ppm, corresponding to *M. formicicum* grown on inorganic medium. The highest value was 157 ppm for *M. barkeri*, also grown on inorganic medium. Additional factors of importance are pressure and static vs. shaking cultures (for enhanced dissolution of gaseous substrates). Atmospheric pressure on Mars is approximately 100 times less than that on Earth, and whether there is movement in subsurface aquifers where life might exist is unknown. Pressure studies indicate that these methanogens grow best at higher pressures (e.g. 300 kPa), and even though shaking of culture tubes allowed for the most rapid growth rates and yields, static cultures incubated horizontally grew quite well.

Also, funding from this NASA-Ames Consortium grant has helped to support the research of a number of students. Those completing Honors projects and the titles of their research are listed below.

Thomson, Daniel R. Effects of Vitamin and Mineral Solutions on the Growth of *Methanobacterium wolfei*. 1995.

Boutzale, Christine L. The Effects of Varying Pressure on the Growth of Two Methanogens *Methanosarcina barkeri* and *Methanobacterium wolfei*. 1995.

Everitt, Melanie D. The Effects of Gas/Liquid Interactions on the Growth of Methane-Producing Bacteria. 1995.

McMahan, Melissa B. The Effects of Reducing Agents on Methanogens. 1995.

Ryan, Cara L. Could Antarctic Methanogens Really be Martians: Characterization of a Psychrophilic Methanogen. 1996.

Brewer, Jim Ed. Varying Pressures and the Resulting Growth Rates in Methanogens. 1996.

Also, one student completed a Howard Hughes Research Project.

Chan, Y-S. Connie. The Effects of Varying Pressure on the Growth of a Methanogen *Methanobacterium wolfei*. 1995.

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Hydrogen Consumption by Methanogens on the Early Earth

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Abstract. It is possible that the first autotroph used chemical energy rather than light. This could have been the main source of primary production after the initial inventory of abiotic organic material had been depleted. The electron acceptor most readily available for use by this first chemoautotroph would have been CO_2. The most abundant electron donor may have been H_2 that would have been outgassing from volcanoes at a rate estimated to be as large as 10^{12} moles/year, as well as from photo-oxidation of Fe^{+2}. We report here that certain methanogens will consume H_2 down to levels as low as 10 ppm with CO_2 as the sole carbon source. The lower limit of pH_2 for growth of methanogens can be understood on the basis that the pH_2 needs to be high enough for one ATP to be synthesized per CO_2 reduced. The pH_2 values needed for growth measured here are consistent with those measured by Stevens and McKinley for growth of methanogens in deep basalt aquifers. H_2-consuming autotrophs are likely to have had a profound effect on the chemistry of the early atmosphere and to have been a dominant sink for H_2 on the early Earth after life began rather than escape from the Earth's atmosphere to space.

1. Introduction

The standard theory for the origin of life postulates that life arose from an abiotically produced soup of organic material (e.g., Miller, 1953; Miller, 1992). The first organism would have therefore been a heterotroph deriving energy from this existing pool of nutrients. This theory for the origin of life is not without competitors (for a review or theories for the origins of life see Davis and McKay, 1996), but has received considerable support from laboratory experiments in which it has been demonstrated that biologically relevant organic materials can be easily synthesized from mildly reducing mixtures of gases (e.g., Chang *et al.*, 1983). The discovery of organics in comets (e.g., Kissel and Kruger, 1987), on Titan (e.g., Sagan *et al.*, 1984), elsewhere in the outer solar system (e.g., Encrenaz, 1986), as well as in the interstellar medium (e.g., Irvine and Knacke, 1989) has further strengthened the notion that organic material was abundant prior to the origin of life.

A key question in this scenario is how life survived after this initial feedstock of organics had been depleted due to unchecked microbial growth. It would have been necessary for life to develop autotrophic

pathways. Margulis (1970) and others (Wachtershauser, 1990; Walker, 1977) have suggested that the first autotrophs used chemical energy rather than photosynthesis. One possible pathway is that expressed in methanogens:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

The early atmosphere of the Earth is thought by some researchers, but not all, to have contained high levels of CO_2, perhaps enough to account for warm surface temperatures despite solar luminosities lower by about 25% (Kasting, 1990; Walker, 1977). Volcanic gases would have provided a source of H_2 estimated to be as large as 1.6×10^{12} moles/yr (Holland, 1978). Additional H_2 would have arisen from photo-oxidation of aqueous Fe^{+2} (Braterman et al., 1983) and diagenesis of organic compounds.

The atmospheric effects of microbial consumption of H_2 could have been profound. By converting H_2 and CO_2 to CH_4, and limiting H_2 levels in the atmosphere, microorganisms would have altered the chemical and radiative properties of the atmosphere. Previous studies have

shown that methanogens can consume H_2 down to levels of 22 ppm when provided with a source of organic material (Lovley, 1985). For the application to the early Earth we must extend these results to purely chemoautotrophic growth.

To determine the lower limit at which chemoautotrophic methanogens can consume H_2 we have conducted tests on several methanogens. In particular we report results for *Methanobacterium wolfei*, *Methanobacterium formicicum*, and *Methanosarcina barkeri*. The *Methanobacterium* species are commonly found in anaerobic digesters and in anaerobic sediments from freshwater environments. *M. barkeri* is found in marine and freshwater mud as well as anaerobic digesters (Stanley, 1989).

2. Materials and Methods

With some modifications, our experimental procedure follows that of Lovley (1985). Cultures were obtained from David R. Boone, Oregon Graduate Institute, Beaverton, OR. All cultures were grown in a standard medium (MS medium) for methanogens as described by

Boone *et al.* (1989) or the same medium without any organic material (Xun *et al.*, 1988). All media were prepared by standard anaerobic techniques (Hungate, 1969) in a Coy Anaerobic Environmental Chamber. Media were autoclaved at 121C for 20 min. Sodium sulfide (2.5%) was added 1 hour before inoculation from sterile, oxygen-free stock solutions (Boone *et al.*, 1989). Cultures (10 ml) were grown under 300 kPa total pressure of hydrogen-carbon dioxide (75:25) in anaerobic pressure tubes (Hungate, 1969), followed by horizontal incubation in environmental shakers at 55C for *M. wolfei* and 37C for *M. formicicum* and *M. barkeri*. Culture optical densities were measured at 660 nm in a Bausch & Lomb Spectronic 20. When each culture reached an optical density of 0.1 to 0.2, the headspace was flushed and replaced with pure CO_2 (300 kPa total pressure). Hydrogen was then added to provide 10,000 to 20,000 ppm in the headspace. To prove the reproducibility of the result, we reinjected H_2 after 48 hours and observed again the return to the lower limit of uptake.

Headspace gas samples (1 ml) were removed at time intervals and analyzed by a Hewlett-Packard model 5890 gas chromatograph with a

thermal conductivity detector at an oven temperature of 40C using argon as the carrier gas.

3. Results

The results for growth on inorganic medium are shown in Figure 1. For all three methanogens considered there is a clear and reproducible limit on the levels of H_2 where uptake occurs. These levels vary among the organisms tested with the lowest value, 10+/-10 ppm, corresponding to *M. formicicum*. The digital resolution of the gas chromatograph was 10 ppm. For this reason any values below 10 ppm were recorded as zero. For *M. formicicum* we observed values that were close to but above 10 ppm (Figure 1, panel c) and we assume that values below the resolution are near 10 ppm. Values below 10 ppm are not plotted in Figure 1 but were used (with an average value of 5 ppm) in computing the minimum H_2 uptake level. The highest value of the H_2 uptake level was 157+/-21 ppm for *M. barkeri*.

In Figure 2 we present the results for growth on organic-supplemented medium. The results are comparable to those obtained on the organic-free medium.

4. Discussion

Our results for heterotrophic growth (Figure 2) can be compared to those of Lovley and colleagues (Lovley, 1985; Lovley and Ferry, 1985; Lovley and Goodwin, 1988). For example, they found that *M. formicicum* in pure culture would take up H_2 to a partial pressure of 6.5 Pa (Lovley and Ferry, 1985; equivalent to 22 ppm in our experiments). Studies on whole sediments (Lovley and Goodwin, 1988) also show uptake of H_2 down to levels of 7-10 nM in solution (equivalent to 3-5 ppm in our experiments).

The lower limit of pH_2 for growth of methanogens can be understood in terms of the Gibbs free energy available from the reduction of CO_2.

$$CO_2(g) + 4H_2(g) \rightarrow CH_4(g) + 2H_2O(l)$$

Using the free energies of formation (Stull *et al.*,1969; Miller and Smith-Magowan,1990) gives

ΔG°_{react} = -31.27 kcal/mole (25C) and ΔH°_{react} = -60.48 kcal/mole

$\log K_{eq} = -21.405 + 13{,}217/T$

ΔG°_{react} (kcal/mole) = -60.48 + 0.09795T

The free energy change is very temperature dependent with

ΔG°_{react} (37C) = -30.12 kcal/mole

and ΔG°_{react} (55C) = -28.37 kcal/mole

The free energy change at pressures different than the standard 1 atm is given by

$\Delta G_{react} = \Delta G^{\circ}_{react} + RT\ln Q$

where $Q = (pCH_4)(a_{H2O})^2/(pCO_2)(pH_2)^4$

The values for *M. barkeri* grown on inorganic medium at 37C are $pCH_4 = 26 \times 10^{-2}$ atm, $a_{H2O} = 1.0$, $pCO_2 = 3.0$ atm and $pH_2 = 4.71 \times 10^{-4}$ atm. This gives a $\Delta G_{react} = -14.64$ kcal/mole. Since the $\Delta G°$ of hydrolysis of ATP is -8.5 kcal/mole, this is enough to synthesize just one ATP, if it is assumed that the overall reduction of CO_2 needs to be irreversible, as is the case for most biosynthetic pathways.

The calculated free energies for the other methanogens are

M. barkeri at 37C, organic medium, $\Delta G_{react} = -14.10$ kcal/mole

M. formicicum at 37C, inorganic medium, $\Delta G_{react} = -7.51$ kcal/mole

M. wolfei at 55C, inorganic medium, $\Delta G_{react} = -8.96$ kcal/mole

The last two ΔG values seem a little low if several kcal/mole of irreversibility are needed, but the $\Delta G°$ of hydrolysis of ATP is rather

dependent on Mg^{+2}, Ca^{+2}, temperature and the ATP/ADP ratio. It is also possible that the ATP is produced by a chemiosmotic pathway from the free energy obtained from the reduction of two CO_2 molecules since chemiosmotic processes are involved in methane production (Thauer, 1990), although this does not seem to be required in this case. It is noted that formate has an equivalent pH_2 of nearly 1 atm (Miller and Smith-Magowan, 1990) so that traces of formate in the medium could provide considerable free energy in addition to that provided by the H_2.

Stevens and McKinley (1995) report that the growth of methanogens in deep basalt aquifers stops at $[H_2]$ <0.02 μmolar. From the solubility of H_2 at 25C (Wilhelm *et al.*, 1977), this corresponds to $pH_2 = 2.6 \times 10^{-5}$ atm. The free energy change cannot be calculated because the pCH_4 is not given for each pH_2 measurement, but these ΔG values are approximately in agreement with those we obtained from growth in culture.

Our results for growth on organic-free medium suggest that consumption of H_2 by methanogens on the early Earth could have been

an important sink for H_2 and source for CH_4. Methanogens can use CO_2 at pH_2 levels many times that of our present atmosphere. These levels are significantly in excess of the threshold for consumption by methanogens. The major non-biological net sink of H_2 is escape to space (Walker, 1977). The rate of escape of total hydrogen depends on the mixing ratio of all hydrogen-containing species. However, since H_2 is not significantly reformed in the atmosphere - its source if outgassing (Kasting *et al.*, 1993) - its loss rate by escape to space is determined by its own mixing ratio. Thus, as methanogens convert H_2 and CO_2 to CH_4 they become the only important net sink for H_2. The CH_4 formed can continue to contribute to the escape of hydrogen from the atmosphere.

Methanogens produce CH_4 as a byproduct. Methane is an important greenhouse gas but because it is rapidly photolyzed, a continuous source is required for it to have played an effective role in maintaining above freezing temperatures on the early Earth against the lower luminosity of the early sun (Caldeira and Kasting, 1992) Conversion of H_2 and CO_2 to CH_4 by methanogens could be such a source. To adequately address these issues requires that photochemical and greenhouse models of the early Earth explicitly include the effects of

microbial consumption of H_2 and production of CH_4. Further experiments focused on the rate of consumption for concentrations above the minimum could elucidate the kinetics of microbial uptake in a way that would allow for incorporation into numerical models.

Methanogenic chemosynthesis can be the basis for a microbial ecosystem. Recently, Stevens and McKinley (1995) have reported on a microbial ecosystem deep within basaltic sediments. Primary productivity is based on methanogens consuming H_2 from solution at levels between 0.05 and 60 uM. The H_2 may come from water reacting with basaltic rocks. This system represents an extant model for methanogenic primary production such as we have hypothesized might have existed on the early Earth.

It has also been suggested that a microbial ecosystem based on H_2 and CO_2 could be the basis for subsurface life on Mars where surface conditions are inhospitable to life (Boston *et al.*, 1992). Subsurface volcanic activity could provide liquid water by melting ground ice and could also be a source of reduced gases such as H_2S and H_2.

Alternatively H_2 could be produced by water reacting with basalt - abundant on Mars - as in the terrestrial example.

Acknowledgements

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Figure Captions

Figure 1. Time course for H_2 utilization by methanogens in three atmospheres of CO_2. At the start of the time course, and again at 48 hours, H_2 was added to the cultures. In all cases the organisms rapidly depleted H_2 levels down to a minimum value of 157+/-21 ppm for *M. barkeri* (panel a); 49+/-21 ppm for *M. wolfei* (panel b); 10+/-10 ppm for *M. formicicum* (panel c). Three time courses are shown for each organism using different symbols.

Figure 2. Same as Figure 1 but with organic medium. Minimum values for H_2 uptake were 121+/-24 ppm for *M. barkeri* (panel a); 61+/-19 ppm for *M. wolfei* (panel b); 12+/-8 ppm for *M. formicicum* (panel c).

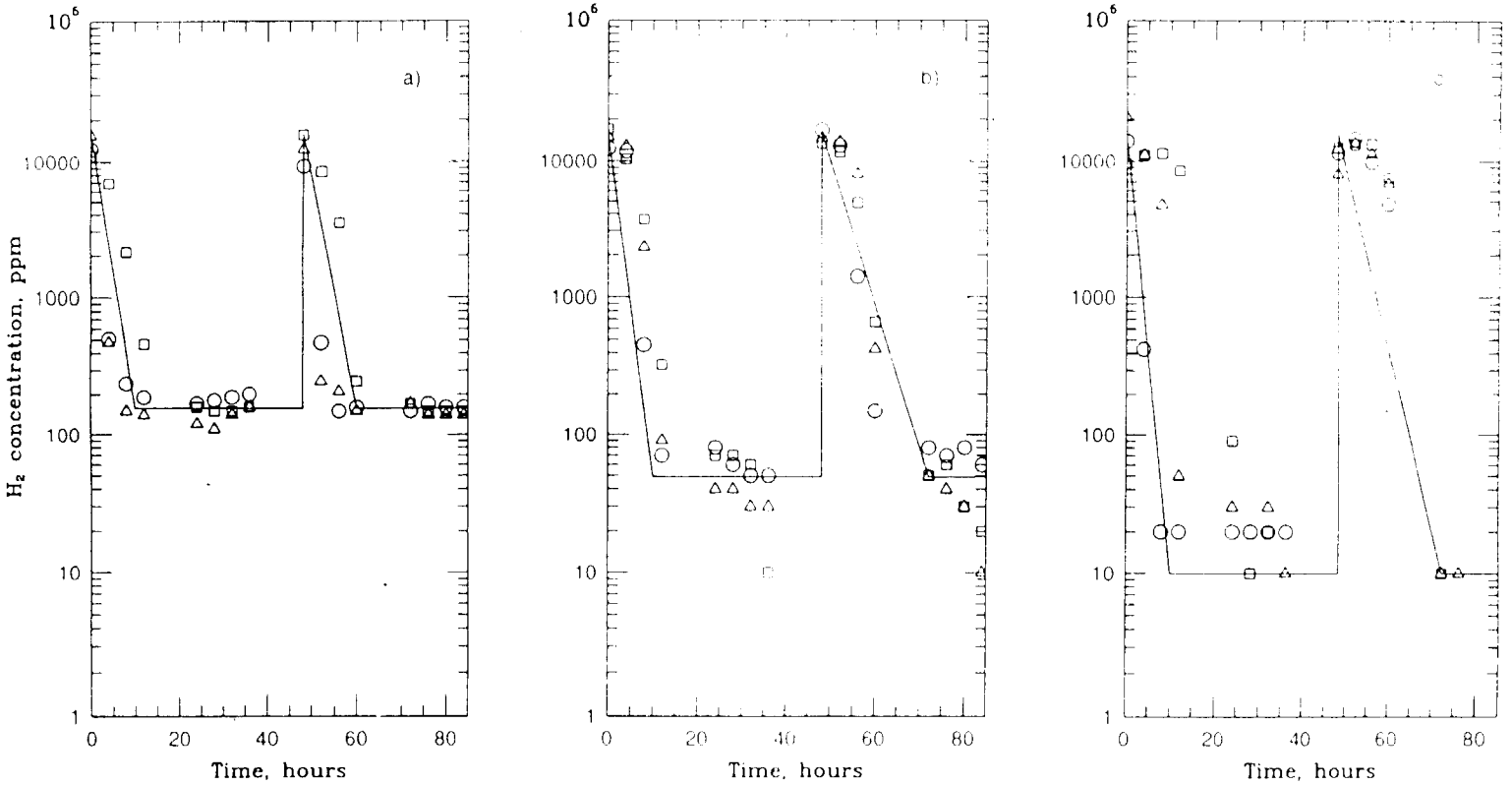

Figure 1, Krai et al.

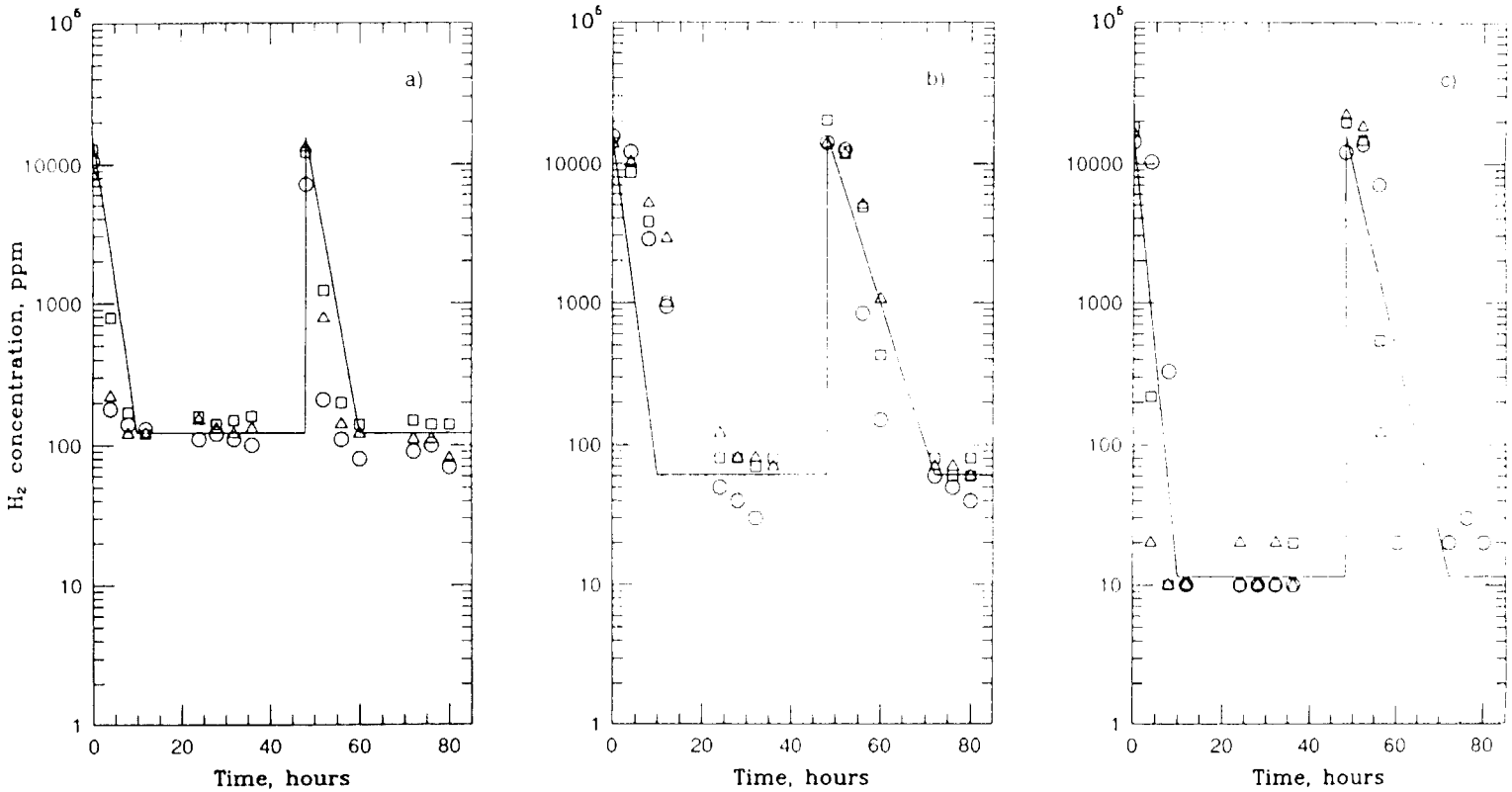

Figure 2, Kral et al.

Grant Proposal

Hydrogen Consumption and Methane Production by Methanogens: Implications for Mars and Early Earth

P.I.: Timothy A. Kral

PROJECT DESCRIPTION

Purpose

The purpose of this research is threefold:

1) We will continue studies to determine the hydrogen threshold of numerous methanogens under varying conditions. If hydrogen is present below the surface of Mars, it will probably exist in very limited quantities. Therefore, determining hydrogen thresholds of methanogens is a significant goal.

2) We will examine the growth of methanogens in an environment where the hydrogen is derived from the interaction of basalt and anaerobic water. With the question of hydrogen below the surface of Mars unresolved, and the fact that Mars contains tremendous amounts of basalt, the recent discovery that basalt and anaerobic water can generate hydrogen gives us an exciting new direction of research to pursue.

3) We will examine the stable carbon isotope partitioning during growth of methanogens under various conditions. At 2.8 billion years ago or earlier, something in the carbon cycle began to allow the incorporation in sediments of organic material extraordinarily depleted in ^{13}C. We want to determine what possible role methanogens may have played on the early Earth.

1. Hydrogen Thresholds

In 1976, the Viking Lander missions analyzed the Martian soil for evidence of life. While the results led to some controversy, most investigators concluded that there was no biological activity at the lander sites (1, 2). But the Viking Landers only examined the surface of Mars where conditions are too dry, too cold, and too oxidizing for life to exist as we know it. It may be possible that liquid water can exist and persist below the surface. There is evidence that liquid water has been present in subsurface aquifers on Mars throughout its history (3). Lifeforms existing below the surface could not obtain their energy from photosynthesis, but rather they would have to utilize chemical energy. Because the Viking Landers found no measurable quantities of organic matter, lifeforms might be limited to oxidation of inorganic matter for energy. Organisms that fall into this category are referred to as chemoautotrophs. Methanogens are chemoautotrophs that consume molecular hydrogen and carbon dioxide and produce methane as a waste product. A potential habitat for existence of methanogens on Mars might be based on a geothermal source of hydrogen, possibly due to volcanic or hydrothermal activity, or the reaction of basalt and anaerobic water (4), carbon dioxide, which is already abundant in the Martian atmosphere, and subsurface liquid water.

Since carbon dioxide is abundant, and molecular hydrogen might be a limiting nutrient, experiments will be designed to determine minimum threshold concentrations of molecular hydrogen under various conditions of temperature, pressure, pH, and nutrient concentrations that will allow for growth of the methanogen tested.

All organisms will be grown in a standard medium (MS Medium) for methanogens (5) or the same medium without organic material (6). Growth medium will be prepared under 95% carbon dioxide and 5% hydrogen in a Coy environmental chamber. A carbon dioxide rather than the standard nitrogen atmosphere is preferable. Medium made in the described atmosphere will be saturated with carbon dioxide resulting in a pH of 6.7 which is ideal for most methanogens. A nitrogen atmosphere would increase the medium pH to greater than 8, resulting in precipitation of medium components and unfavorable conditions for growth. (pH studies will be described later.) The 5% hydrogen is necessary for the oxygen-removing palladium catalysts to work. (Methanogens are strict anaerobes and will be poisoned by molecular oxygen.) The anaerobically-prepared medium will be added to growth vessels in the anaerobic chamber. Each growth vessel will be sealed with a butyl rubber stopper and a crimped aluminum cap. Outside of the anaerobic chamber, the gas phase in each

vessel will be replaced by100% hydrogen by the use of a gassing manifold. The combination of the hydrogen headspace gas along with a carbon dioxide-saturated liquid medium will result in a combination of substrates ideal for methanogenic growth. In experiments where cultures need to be repressurized, a 75% hydrogen: 25% carbon dioxide mixture will serve the same purpose. At least one hour prior to inoculation, a sterile sodium sulfide solution will be added to each culture tube to eliminate any residual molecular oxygen (5). A sterile 3cc plastic syringe will be used to inoculate each vessel. Growth will be monitored spectrophotometrically using a Spectronic 20 at 660nm. When each culture reaches an optical density of 0.1 to 0.2, the headspace will be flushed and replaced with pure carbon dioxide (300 kPa total pressure). Hydrogen will then be added to provide 10,000 to 20,000 ppm in the headspace. Headspace gas samples (1ml) will be removed at time intervals and analyzed by a Hewlett Packard model 5890 gas chromatograph with a thermal conductivity detector at an oven temperature of 40C using argon as the carrier gas.

Previous studies have shown that methanogens can consume H_2 down to levels of 22 ppm when provided with a source of organic material (7).

Thus far, research in our laboratory has resulted in the submission of a manuscript entitled "Hydrogen Consumption by Methanogens on the Early

Earth" (see attached manuscript). Besides myself, the authors include Keith Brink, graduate student, Stanley Miller who was responsible for the thermodynamic calculations and ensuing discussion, and Chris McKay, our research collaborator from NASA Ames Research Center, Moffett Field, CA. Even though the original impetus for the research dealt with the possibility of life on Mars, the results have significant implications for early life on Earth.

To determine the lower limit at which chemoautotrophic methanogens can consume H_2 we have conducted tests on several methanogens. In the manuscript we report results for *Methanobacterium wolfei*, *Methanobacterium formicicum*, and *Methanosarcina barkeri*. For all three methanogens considered there is a clear and reproducible limit on the levels of H_2 where uptake occurs. For growth on inorganic medium, these levels vary among the organisms tested with the lowest value, 10+/-10 ppm, corresponding to *M. formicicum*. *M. wolfei* demonstrated a minimum value of 49+/-21 ppm, while the highest value of the H_2 uptake level was 157+/-21 ppm for *M. barkeri*. Growth on organic medium resulted in values of 121+/-24 ppm for *M. barkeri*, 61+/-19 ppm for *M. wolfei*, and 12+/-8 ppm for *M. formicicum*.

Our results for heterotrophic growth can be compared to those of Lovley and colleagues (7, 8, 9). For example, they found that *M. formicicum* in pure culture would take up H_2 to a partial pressure of 6.5 Pa (8; equivalent to 22 ppm in our experiments). Studies on whole sediments (9) also show uptake of H_2 down to levels of 7-10 nM in solution (equivalent to 3-5 ppm in our experiments).

The calculated free energies for the methanogens are

M. barkeri at 37C on inorganic medium, ΔG_{react}=-14.64 kcal

M. barkeri at 37C on organic medium, ΔG_{react} = -14.10 kcal

M. formicicum at 37C on inorganic medium, ΔG_{react} = -7.51 kcal

M. wolfei at 55C on inorganic medium, ΔG_{react} = -8.96 kcal

The last two ΔG values seem a little low if several kcal of irreversibility are needed, but the $\Delta G°$ of hydrolysis of ATP is rather dependent on Mg^{+2}, Ca^{+2}, temperature and the ATP/ADP ratio. It is also possible that the ATP is produced by a chemiosmotic pathway from the free energy obtained from the reduction of two CO_2 molecules, although this does not seem to be required in this case. It is noted that formate has an equivalent pH_2 of nearly 1 atm (10) so that traces of formate in the medium could provide considerable free energy in addition to that provided by the H_2.

Following submission of this manuscript, hydrogen thresholds for two other methanogens have been determined. *Methanococcus maripaludis* demonstrated a threshold of 27+/-11 ppm in organic medium and 30+/-12 ppm in inorganic medium. *Methanococcus voltae* demonstrated a threshold of 97+/-28 ppm in organic medium. We have been unable to grow *M. voltae* in inorganic medium so far. The free energy changes are -12.21, -12.45, and -13.06 kcal, respectively.

Additionally, the hydrogen threshold of a psychrophilic methanogen isolated from Ace Lake in Antarctica is being determined. This organism, which has been maintained at 7C, is currently demonstrating a hydrogen threshold of no greater than 140 ppm. Of course, this organism is very interesting to us because of the correlation between its cold-loving characteristic and the extremely low temperatures on Mars. A complete characterization of this organism is in progress.

In order to determine the effects of pH on both growth and hydrogen threshold, medium will be altered by the addition of hydrochloric acid or sodium hydroxide. Molecular nitrogen can be used to increase the pH, but achieving a predetermined stable pH is difficult. The pH of the unaltered medium is 6.7. The pH will be adjusted in 0.5 pH intervals. Initial results indicate that *M. wolfei* prefers to grow in slightly acidic rather than basic

medium. The organism generated 19% methane at pH 6.7, 15% at pH 6.2, and 7.8% at pH 5.7. At pH levels of 7.2 and 7.7, *M. wolfei* generated only 0.69% and 0.06% methane, respectively.

Pressure experiments will also be conducted. A gassing manifold with an oxygen scrubber will be used to deliver pressures of 20 (-80 relative to ambient pressure), 100, 200, and 300 kPa. All of the methanogens tested so far prefer to grow at high pressure. (These results are not surprising due the the low solubility of H_2.) Growth is greatest at 300 kPA, followed by 200 kPa, and then100 kPa. These organisms have not demonstrated growth in preliminary experiments at 20 kPa so far.

Temperature experiments have demonstrated the obvious, maximal methane production at the optimal temperature. In addition, and maybe not so obvious, growth at higher or lower than optimal temperatures has resulted in variable results, depending on the organism. As an example, *M. maripaludis* which grows optimally at 37C produced a fair amount of methane at 27C, but very little at 55C. *M. formicicum*, again with optimal growth at 37C, produced very little methane at either 27C or 55C.

Experiments to determine the requirement for or stimulatory effects of various mineral supplements are also underway. Very preliminary results with *M. wolfei* indicate that aluminum seems to be required whereas the

requirement for selenium is doubtful. This study also includes copper, molybdenum, nickel, cobalt, iron, tungsten, boron, manganese, and zinc.

Once we get a handle on the optimal growth conditions (temperature, pH, pressure, and nutrients) and the effects of altering those conditions on the growth of selected methanogens, we will begin to investigate the hydrogen thresholds under those altered conditions.

2. Geochemically produced hydrogen

In the October 20, 1995 issue of Science, Stevens and McKinley (4) reported on the existence of bacterial communities in deep crystalline aquifers within the Columbia River Basalt Group. Autotrophic microorganisms outnumbered heterotrophs, and stable carbon isotope measurements implied that autotrophic methanogenesis dominated this ecosystem and was coupled to the depletion of dissolved inorganic carbon. Stevens and McKinley presented evidence for an active, anaerobic subsurface lithoautotrophic microbial ecosystem that appears to derive energy from geochemically produced hydrogen. Thus, this ecosystem should persist independently of photosynthetic products. They hypothesized that H_2O reduction to produce H_2, driven by iron in ferromagnesian silicates, could serve as the abiotic energy source. In order to produce direct evidence for H_2 production from basalt-water reactions,

they added steel-free crushed basalt to pH buffered water in sealed tubes under strictly anaerobic sterile conditions in the dark. Rapid H_2 evolution occurred. The basalt was prepared free of steel because steel contamination from drill cuttings may react with water to generate H_2. In order to determine if basalt water reactions alone could support a microbial community, they prepared a series of culture tubes containing sterile crushed basalt and ground water. Microorganisms proliferated, and with increasing time, autotrophs maintained high numbers while heterotrophs declined.

Stevens and McKinley suggest that in addition to providing a model for how surface organisms could have lived on Earth before the evolution of photosynthesis and the development of an oxidizing atmosphere about 2.8 billion years ago, that a model is provided for the existence of extant life on Mars because basalt, liquid water, and bicarbonate are believed to be present in the Martian subsurface.

In our laboratory, we have conducted a very preliminary study on hydrogen generation catalyzed by basalt. One gram of steel-free pulverized basalt was added to 10 ml of MS medium under anaerobic conditions. The medium had been saturated with carbon dioxide. Initial measurements

indicated no H_2 present. After one week of static incubation at room temperature, approximately 3000 ppm H_2 were detected.

Future experiments will include different amounts of basalt in different types of media under varying conditions of pH, pressure, temperature, and nutrients as described previously. The goal is to grow and characterize different methanogens on basalt-anaerobic water derived hydrogen under the stated conditions. Another interesting observation reported by Stevens (personal communication) is that the amount of H_2 produced is greater in the presence of microorganisms than in a sterile buffered solution. This observation would seem to add relevance to our proposal to examine this phenomenon under the varying conditions described with different methanogens. (Todd Stevens, who I'm told is also submitting an Exobiology proposal, and I have briefly discussed the idea of collaborative research.)

3. Stable carbon isotope fractionation

Biochemical incorporation and subsequent biochemical processing entail sizable isotopic effects as a result of both thermodynamic and kinetic fractionations that occur during metabolic and biosynthetic reactions (11). Such fractionations are due for the most part to kinetic isotope effects inherent in enzymatic activity. As these fractionations can be preserved in

sediments with minor alteration, these biochemical processes have exerted an impact on the long-term biogeochemical cycles of the respective elements that can be traced through geological time (11).

Carbon dioxide found in the atmosphere contains about 1.1% of the heavier carbon isotope ^{13}C and 98.9% ^{12}C. Many organisms discriminate against ^{13}C in ways that reflect their metabolism and the environment. Isotopic compositions are measured by means of a mass spectrometer. The output of the spectrometer is a ratio and is usually expressed as $\delta^{13}C$, measured in units per mil ($^{o}/_{oo}$). However, absolute isotope ratios are difficult to obtain. It is usually adequate to give $\delta^{13}C$ values relative to a standard. The generally used standard is PDB (belemnite from the Pee Dee Formation in South Carolina). The ratio for PDB is $^{13}C/^{12}C = 0.01124$ (12). As biological carbon fixation discriminates against ^{13}C, the $\delta^{13}C$ values of biosynthesized cell material are more negative than those of the carbon substrate. Atmospheric carbon dioxide has a $\delta^{13}C = -7\ ^{o}/_{oo}$ while marine bicarbonate has a $\delta^{13}C = -0.5 +/-1\ ^{o}/_{oo}$ (11). The gross average for sedimentary organics of all ages is approximately $-25^{o}/_{oo}$ (11).

So why are we interested in carbon fractionation? At 2.8 billion years ago or earlier, something in the carbon cycle began to allow the incorporation in sediments of organic material extraordinarily depleted in

^{13}C (13). This phenomenon is widespread with reported isotopic compositions as low as -50°/$_{oo}$ (11, 13). One model that has been proposed to explain this phenomenon involves biogenic methane because of its unique extraordinary levels of ^{13}C depletion (13). *Methanogenium thermoautotrophicum* displays discrimination ranging from +6 to -41°/$_{oo}$, in response to the concentration of available CO_2. At low CO_2 concentrations, ^{13}C is actually enriched. This discrimination is also temperature dependent (12, 14). In general, methane generated by methanogenic microorganisms by reduction of CO_2 by H_2 is usually extremely enriched in ^{12}C, with $\delta^{13}C$ values often reaching -60°/$_{oo}$ or lower (4, 11). A lesser fractionation, -20 to -40°/$_{oo}$, is seen when methanogens grow on acetate (4).

So the methanogens may have been major players in the late Archean. We are interested in analyzing carbon isotope partitioning characteristics as they relate to variations in environmental conditions. There are not many studies reporting the effects of environmental conditions on partitioning in methanogens. However, effects in plants have been described by O'Leary (12). Intraspecies variations have been observed in several C_3 and C_4 plants with variations up to 3% (15). The effects of nutrients have been reported for timothy grass (16). Well-nourished plants showed more positive $\delta^{13}C$ values than did plants deficient in

nitrogen and/or potassium. As mentioned earlier, temperature may have an effect on partitioning. Troughton (15) reported that $\delta^{13}C$ values become slightly more negative with increasing temperature in several C_3 and C_4 plants. The bottom line is that environmental factors do affect carbon isotope partitioning to some extent in plants. We want to expand the picture to methanogens (beyond the little that has been published) in order to determine what possible role they may have played on the early Earth.

Because we do not have the mass spectrometer required for the fractionation analysis, the procedure will be simple for us. We will grow the various methanogens under the environmental conditions (temperature, pH, pressure, and nutrients) described previously, remove aliquots of headspace gas at predetermined time intervals, and transfer them to sealed tubes containing pure nitrogen. The sample tubes will be shipped to Craig Cook, Department of Biology, University of Utah, Salt Lake City, for stable carbon isotope fractional analysis of the methane. Results from these analyses should give us detailed information on effects of environment on carbon fractionation in methanogens, and possibly a greater understanding of the role that methanogens may have played on the early Earth.

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